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ORIGINAL PAPER

Variations in the chemical profile and biological activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest times

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Abstract This study investigates the variations in the chemical profile, free radical scavenging, antioxidant and gastroprotective activities of licorice extracts (LE) from plants harvested during the months of February to November. Correlations between biological properties and the chemical composition of LE were also investigated. The results showed that the total contents of phenols, flavonoids and tannins in LE varied at different harvest times. Liquiritin and glycyrrhizin, the major components of LE, varied in the range of 28.65–62.80 and 41.84–114.33 mg g^{-1}, respectively. The relative proportion of glycyrrhizin derivative (3), glabridin (4), glabrene (5) and liquiritigenin derivative (6), varied in the range of 0.88–11.38 %, 1.86–10.03 %, 1.80–18.40 % and 5.53–16.31 %, respectively. These fluctuations correlated positively with changes in the antioxidant and free radical scavenging activities of licorice. In general, the samples from May and November showed the most favorable free radical scavenging and antioxidant effects, whereas the best gastroprotective effect was in May. Liquiritin and glycyrrhizin, the major constituents in the February and May LE, appeared to contribute to the superoxide radical scavenging and gastroprotective effects. Glabridin and glabrene, the compounds with the highest relative proportion in the November LE, accounted for the antioxidant and DPPH scavenging activities of licorice. It is concluded that the chemical profile of licorice quantitatively varied at different harvest times and these fluctuations determined changes in its bioactivities. These data could pave the way to optimize harvesting protocols for licorice in relation with its health-promoting properties.

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Introduction

Licorice, the dry roots of *Glycyrrhiza glabra* L. (Fabaceae), is considered one of the oldest and most widely used herbs around the world (Isbrucker and Burdock 2006). *Glycyrrhiza glabra* is a perennial herb native to the Mediterranean region, and the Middle East that is now widely cultivated throughout Europe (Nassiri-Asl et al. 2007). Licorice has been traditionally used in herbal medicines for its emollient, antitussive and gastroprotective properties (Sabbioni et al. 2005). Glycyrrhizin and flavonoids, such as liquiritin, isoliquiritin and their aglycones, have been reported as the major constituents of licorice and they are perceived as the active principles responsible for its pharmacological efficacy (Zhang and Ye 2009). Modern pharmacological studies have shown that licorice possesses a variety of bioactivities, including antiinflammatory, antioxidant, immunostimulating, antiviral, antiulcerogenic, anticarcinogenic and hepatoprotective activities (Nassiri-Asl and Hosseinzadeh 2008). Besides medicinal usage, the licorice extract has GRAS (generally regarded as safe) status as a flavoring and sweetening agent for tobaccos, chewing gums, candies, toothpaste, and beverages (Isbrucker and Burdock 2006). The growing use of licorice extracts as active ingredients in cosmetic and personal care products is also worth noting.

Recently, the demand for licorice has been increasing, while the availability of wild licorice has declined (Yamamoto and Tani 2006). The promotion of licorice cultivation as an additional and stable source of the medicinal plant requires additional studies to determine the influence of enviromental factors on its chemical content and biological properties. The harvesting of licorice root occurs in the autumn of its third or fourth year of growth (Isbrucker and Burdock 2006), which is mainly dependent on the total yield of the plant rather than on its chemical composition. Botanicals have been reported to have variable chemical compositions due to variations in soil types and climates as well as to harvest times or growing periods (Incerti et al. 2009; Yesil-Celiktas et al. 2007; Çyrak et al. 2007). The influence of harvest times and their associated enviromental factors on the chemical and biological conditions of licorice have been largely unexplored, except for a few studies revealing seasonal variation in glycyrrhizin and isoliquiritin contents (Hayashi et al. 1998), and the influence of UV radiation on glycyrrhizin biosynthesis (Afreen et al. 2005).

The present study is aimed to investigate the variation in the chemical composition and free radical scavenging, antioxidant and gastroprotective activities of extracts of licorice collected at different harvest times during a 1-year period.

Materials and methods

Chemicals

N-Methylphenazonium methyl sulfate (PMS), β-nicotinamide adenine dinucleotide reduced (NADH), nitrotetrazolium blue chloride (NBT), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), hide powder, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40) and standards (glycyrrhizin and quercetin) were obtained from Sigma-Aldrich (Steinheim, Germany). Standard liquiritin was purchased from Wuhan Sunrise Technology Development Co., Ltd. (Hong Kong, China) and β-carotene was from Koch-light Laboratories Ltd. (England). Folin–Ciocalteu phenol reagent, aluminum chloride hexahydrate, formalin, absolute ethanol and sodium carbonate were from Merck (Darmstadt, Germany). HPLC grade solvents were also from Merck. Ultrapure water from the Milli-Q RG system (Millipore, Molsheim, France) was used, and lansoprazole, captopril and flufenamic acid were purchased from Sigma Chemical Co. (USA).

Animals

Male Swiss albino mice were purchased from the Instituto de Salud Pública de Chile, Santiago. The mice weighing 30 ± 3 g were used and they were fed on the certified Champion diet with free access to water under standard conditions of 12-h dark–light period, and 22 ± 2 °C room temperature.

Sample collection and preparation of extracts

The thickened roots of 4-year-old cultivated *Glycyrrhiza glabra* L. were collected in the middle of February, May, August and November of 2008 in the Botanical Garden of the Faculty of Horticulture, Mendel University of Agriculture and Forestry, Czech Republic at 164 m above sea level. Three samples of *G. glabra* from each harvest time were used. The genetic resource was identified with the code 0001. The mean values for maximum and minimum temperature (°C) for the months of February, May, August and November 2008 were 7.3 °C/−0.8 °C; 20.5 °C/9.5 °C; 25.7 °C/14.1 °C and 9 °C/4.1 °C, respectively. The mean values of global and UV radiation for February, May, August and November of 2008 were 6.73 and 39.57; 20.21 and 247.09; 17.77 and 250.97; and 3.38 and 22.09 MJ m^{-2}, respectively (data provided by the Solar and Ozone Observatory of the Czech Hydrometeorological Institute). The plant material was dried at 40 °C in an oven and then

ground to fine powders (mesh size 20). A precisely weighed amount (1.50 g) of the powder was extracted under reflux with 150 ml of 80 % methanol at 60 °C for 6 h. Extractions were carried out in triplicate and materials obtained were filtered over Whatman No. 1 paper and methanol was then removed under reduced pressure by a rotary evaporator at 35 °C. The resulting aqueous extract was lyophilized and the extraction yield was calculated based on the dry weight of the licorice. These extracts (LE) were assessed for their chemical profile and biological activities.

Determination of total content of phenols and tannins

The total phenol (TP) content was determined using the Folin–Ciocalteu procedure (Cheel et al. 2007). Quantification was plotted on a standard curve of gallic acid. The total tannin (TT) content was determined as above, after the removal of tannins by their adsorption on an insoluble matrix (hide powder). The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight of extract. Data are reported as mean ± standard deviation (SD) to an accuracy of three replicates.

Determination of total content of flavones and flavonols

The total flavone and flavonol (TF) content was determined by the aluminum chloride method (Cheel et al. 2007). Quercetin was used as a reference for the calibration curve. The results were expressed as milligrams of quercetin equivalents (QE) per gram of dry weight of extract. Data are reported as mean ± SD to an accuracy of three replicates.

HPLC–DAD analysis

HPLC analysis was performed, using a Jasco PU-2089 pump equipped with a Jasco MD-2015 diode array detector (DAD), and chromatographic separations were performed on a LiChrospher RP-18 column (4.0 × 250 mm i.d., 5 µm). Major components of LE were identified by comparison of their retention times and UV spectra with those of authentic standards (liquiritin and glycyrrhizin). These compounds were quantified in the extracts using calibration curves (Cheel et al. 2010). Given that no standard compounds for other constituents of LE were available, their relative proportions were estimated based on the percentage of the total peak area in the HPLC chromatogram.

HPLC–ESI–MS analysis

Mass spectra were obtained using a LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). The HPLC separation was done using a Waters SymmetryShield RP18 column (3.5 µm; 2.1 × 100 mm), at 40 °C under a flow rate of 175 µl min^{-1}. The solvent gradient was similar to those for HPLC–DAD analyses. Experiments were performed in negative ion mode. The range for scanning was 200–600 and the capillary temperature was set at 350 °C. High spray voltage was set at 4,500 V. Nitrogen was used as both sheath and auxiliary gas at a flow rate of 25 arbitrary units (approximately 0.56 l min^{-1}) for sheath gas and 5 arbitrary units (approximately 1.5 l min^{-1}) for auxiliary gas. MS/MS were carried out using helium as the target gas, and the collision energy was set at 50–60 %. Identifications were achieved on the basis of the ion molecular mass, MS/MS, and UV–Visible spectra. Data acquisition and processing were carried out using Xcalibur Software (Version 1.2).

Free radical scavenging activity

DPPH free radical scavenging activity

The capacity of samples to scavenge DPPH was assessed as previously reported (Cheel et al. 2007). Samples were assayed at concentrations in the range of 10–100 µg ml^{-1}. The values are reported as mean ± SD of three determinations. Quercetin was used as a reference compound. The percentage of DPPH scavenging effect was calculated as follows:

$$\text{DPPH scavenging activity}\,(\%) = [(E - S)/(E)] \times 100,$$

where $E = A - B$ and $S = C - D$, A is the absorbance of the control, B the absorbance of the control blank, C the absorbance of the sample and D is the absorbance of the sample blank.

Superoxide anion radical scavenging activity

Superoxide radicals were generated by the NADH/PMS system according to a previously described procedure (Valentão et al. 2001). Quercetin was used as a reference compound. Values are presented as mean ± SD of three determinations. The percentage of the superoxide anion scavenging effect was calculated as follows:

$$O_2^{\cdot-}\ \text{scavenging activity}\,(\%) = [(E - S)/(E)] \times 100,$$

where $E = A - B$ and $S = C - D$; A is the absorbance of the control, B the absorbance of the control blank, C the absorbance of the sample and D is the absorbance of the sample blank.

Antioxidant activity

Inhibition of β-carotene-linoleate bleaching

The antioxidant activity of samples was evaluated using the β-carotene/linoleic acid model system (Suja et al. 2005).

Samples were evaluated at concentrations in the range of 5–200 μg ml^{-1}. Quercetin was used as a reference compound. The antioxidant activity of samples was based upon three different parameters, namely antioxidant activity (A_A), oxidation rate ratio (R_{OR}) and antioxidant activity coefficient (C_{AA}). Antioxidant activity (A_A) was determined as a percentage of inhibition relative to the control sample.

$$A_A = [(R_{control} - R_{sample})/(R_{control})] \times 100,$$

where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. The bleaching rates ($R_{control}$ and R_{sample}) were calculated according to first-order kinetics:

$$R_{control\ or\ samples} = \ln(A_t/A_x) \times 1/t,$$

where ln is the natural log, A_t is the initial absorbance at 470 nm at $t = 0$ and A_x is the absorbance at 470 nm at $t = 10$, 20, 30 min.

The oxidation rate ratio (R_{OR}) was calculated by

$$R_{OR} = R_{sample}/R_{control},$$

where R_{sample} and $R_{control}$ are as described earlier.

The antioxidant activity coefficient (C_{AA}) was calculated using:

$$C_{AA} = [(A_{s(180)} - A_{c(180)})/(A_{c(0)} - A_{c(180)})] \times 1000,$$

where $A_{s(180)}$ is the absorbance of the sample containing antioxidant at $t = 180$ min, $A_{c(180)}$ is the absorbance of the control at $t = 180$ min and $A_{c(0)}$ is the absorbance of the control at $t = 0$ min.

Hypochlorous acid scavenging activity

The capacity of samples to scavenge hypochlorous acid (HOCl) was measured with the taurine chlorination assay (Van Antwerpen et al. 2008a). Final concentrations of LE in the assay media were in the range 100–300 μg ml^{-1}, whereas the compounds 1 and 2 were at 12 and 25 μg ml^{-1}. Captopril (4 μg ml^{-1}) was used as a reference compound. The results were expressed as mean ± SD to an accuracy of three determinations.

Inhibition of myeloperoxidase-chlorinating system

The measurement of the inhibition of the myeloperoxidase (MPO)-chlorinating activity was performed in a 96-well plate, as previously described (Van Antwerpen et al. 2008b). Final concentrations of LE in the assay media were in the range 100–300 μg ml^{-1}, whereas the compounds 1 and 2 were at 12 and 25 μg ml^{-1}. Flufenamic acid (0.3 μg ml^{-1}) was used as a reference compound. The results were expressed as mean ± SD to an accuracy of three determinations.

Gastroprotective activity

The gastroprotective activity of the samples was assessed in the ethanol-induced lesion model (Areche et al. 2008). Mice were randomly placed into groups of seven or eight and food was withheld for 24 h with free access to water prior to the experiment. Fifty minutes after oral administration of the extracts (5, 25, 50 mg kg^{-1}), compounds 1 and 2 (5, 10, 25 mg kg^{-1}), lansoprazole (20 mg kg^{-1}) or saline (vehicle), all groups were orally treated with 0.2 ml of a solution containing 99.5 % ethanol for gastric lesion induction. The ulcerated stomachs were fixed in 5 % formalin for 30 min and opened along the greater curvature. The gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black-red lines, parallel to the long axis of the stomach similar to the ethanol-induced lesions in rats. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions. All animal procedures were carried out in accordance with Canadian Council on Animal Care and after ethics committee approval of the University of Chile.

Statistical analysis

To determine whether there was any difference between values, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were applied with the level of significance at $P < 0.05$. After the Bartlett's test for homogeneity of variance, values obtained from the gastroprotective assays were analyzed by ANOVA followed by Dunnett's test with the level of significance at $P < 0.01$. To assess the relationship between variables, Pearson's correlation coefficients were calculated with 95 % confidence. The GraphPad Prism 5 software was used to analyze the data.

Results and discussion

Extraction yield

The extraction yield of licorice from February, May, August and November samples were 256.5, 214, 260 and 260 g kg^{-1}. The lowest value was from the LE obtained from plant at the pre-flowering stage, whereas the highest extraction yields were from both the LE of the plant at the fruit-set and vegetative stages. The extract yield did not exhibit regular changes related to plant growth and course

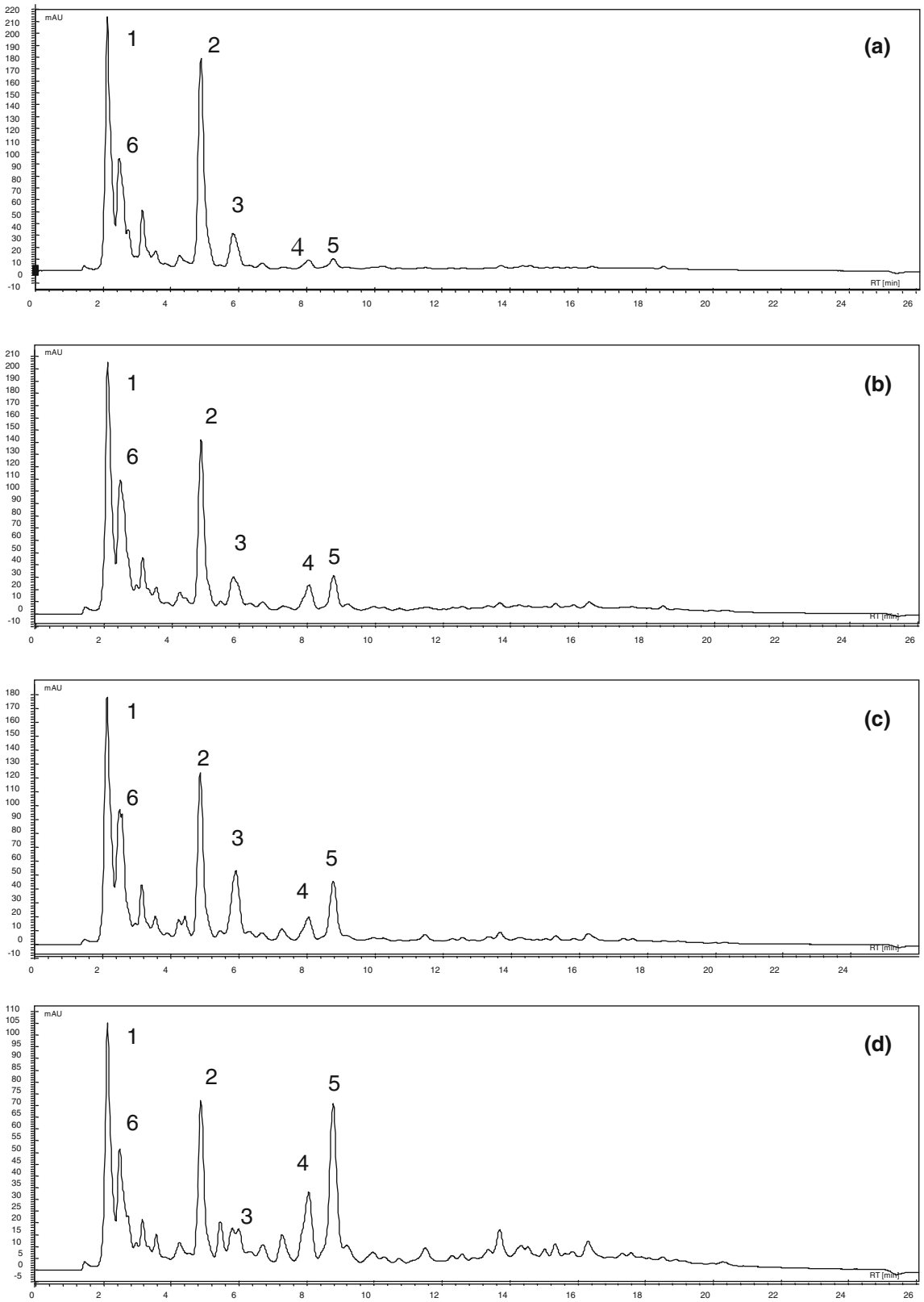

Fig. 1 HPLC chemical profile of licorice extract (LE) from *G. glabra* harvested during the months of February (**a**), May (**b**), August (**c**) and November (**d**). Detection at 254 nm. Peaks: (*1*) liquiritin; (*2*) glycyrrhizin; (*3*) glycyrrhetin glycoside; (*4*) glabridin; (*5*) glabrene and (*6*) liquiritigenin glycoside

Table 1 Contents (mg g^{-1}) of total phenols (TP), total flavonoids (TF), total tannins (TT), liquiritin (1) and glycyrrhizin (2) in licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times

Components	Months			
	February	May	August	November
TP	72.01 ± 0.51	88.43 ± 0.49	99.86 ± 0.72	107.93 ± 0.74
TF	18.42 ± 0.49	25.51 ± 0.09	44.20 ± 0.64	35.03 ± 0.65
TT	4.80 ± 0.24	12.78 ± 0.72	7.86 ± 0.12	9.76 ± 0.30
1	61.68 ± 2.24[a]	62.80 ± 2.13[a]	54.26 ± 1.52	28.65 ± 0.10
2	114.33 ± 2.48	88.87 ± 1.86	76.76 ± 0.93	41.84 ± 0.16

Values followed by the same letter in the same row are not significantly different (Tukey's test, $P < 0.05$, $n = 3 \pm$ SD)

Table 2 Relative proportion (area % HPLC) of compounds 3, 4, 5 and 6 in licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times

Components	Months			
	February	May	August	November
3	7.12 ± 0.08	5.84 ± 0.01	11.38 ± 0.70	0.88 ± 0.02
4	1.86 ± 0.02	4.78 ± 0.01	4.26 ± 0.27	10.03 ± 0.06
5	1.80 ± 0.06	5.32 ± 0.01	9.02 ± 0.61	18.40 ± 0.05
6	10.14 ± 0.23	16.31 ± 0.35	12.82 ± 0.82	5.53 ± 0.10

Values in the same row are significantly different (Tukey's test, $P < 0.05$, $n = 3 \pm$ SD). Glycyrrhetin glycoside (3), glabridin (4), glabrene (5) and liquiritigenin glycoside (6)

time. Similar findings were reported for *Juniperus* species (Adams 1987) and for Turkish oregano (Ozkan et al. 2010).

HPLC–DAD and HPLC–ESI–MS analysis

There was no qualitative variation in the HPLC profile of LE from plants collected at different harvest times (Fig. 1). Liquiritin (1) and glycyrrhizin (2), the two major peaks in the HPLC chromatogram, varied significantly across harvest times in the ranges of 28.65–62.80 and 41.84–114.33 mg g^{-1}, respectively (Table 1). The UV spectra of compounds 3 and 6 in HPLC–DAD chromatogram were similar to those of glycosides of glycyrrhetin and liquiritigenin, respectively (Kitagawa et al. 1998; Zeng et al. 1990). Because the UV spectra from the HPLC–DAD analysis was insufficient to identify peaks 4 and 5, it was necessary to conduct HPLC–MS analysis. The MS spectra of peaks 4 and 5 showed the deprotonated molecules $[M–H]^-$ (*m/z* 323) and $[M–H]^-$ (*m/z* 321), respectively. The negative ion mode ESI–MS^2 spectra of the peak 4 revealed two abundant ions at *m/z* 135 and *m/z* 201, whereas the peak 5 was characterized by a predominant ion at *m/z* 306 and much smaller ions (Online Resource 1), that were consistent with those previously reported in literature for glabridin and glabrene, respectively (Simons et al. 2009). The relative proportions (area % HPLC–DAD) of 3, 4, 5 and 6 significantly varied across harvest times in the range of 0.88–11.38, 1.86–10.03, 1.80–18.40 and 5.53–16.31 %, respectively (Table 2). The chemical structures of the LE compounds are shown in Online Resource 2.

In previous studies, glycyrrhizin was reported to constitute 1–7 % of hydroalcoholic extracts of licorice from different origins (Kondo et al. 2007), whereas the liquiritigenin glycosides were shown to constitute 1.6 % of licorice extract (Kitagawa et al. 1998). The contents of glabridin and glabrene in licorice extracts were estimated at 11.6 and 2.8 %, respectively (Vaya et al. 1997; Okada et al. 1989). In the present study, liquiritin and glycyrrhizin showed a similar variation pattern, which suggests that their biosynthesis are similarly regulated. The biosynthesis of 1 and 2 seems to be favored in the cooler harvest time (February), which corresponds to the vegetative stage of *Glycyrrhiza glabra* (Chrtková 1995). The variation pattern of 2 over harvest times was the opposite of that previously reported for the same compound in licorice cultivated in Turkey (Hayashi et al. 1998), but in agreement with that of other saponins such as gingsenosides from roots of *Panax notoginseng* (Dong et al. 2003). In contrast to an earlier investigation (Afreen et al. 2005), in the present study, the variation pattern of glycyrrhizin content across harvest

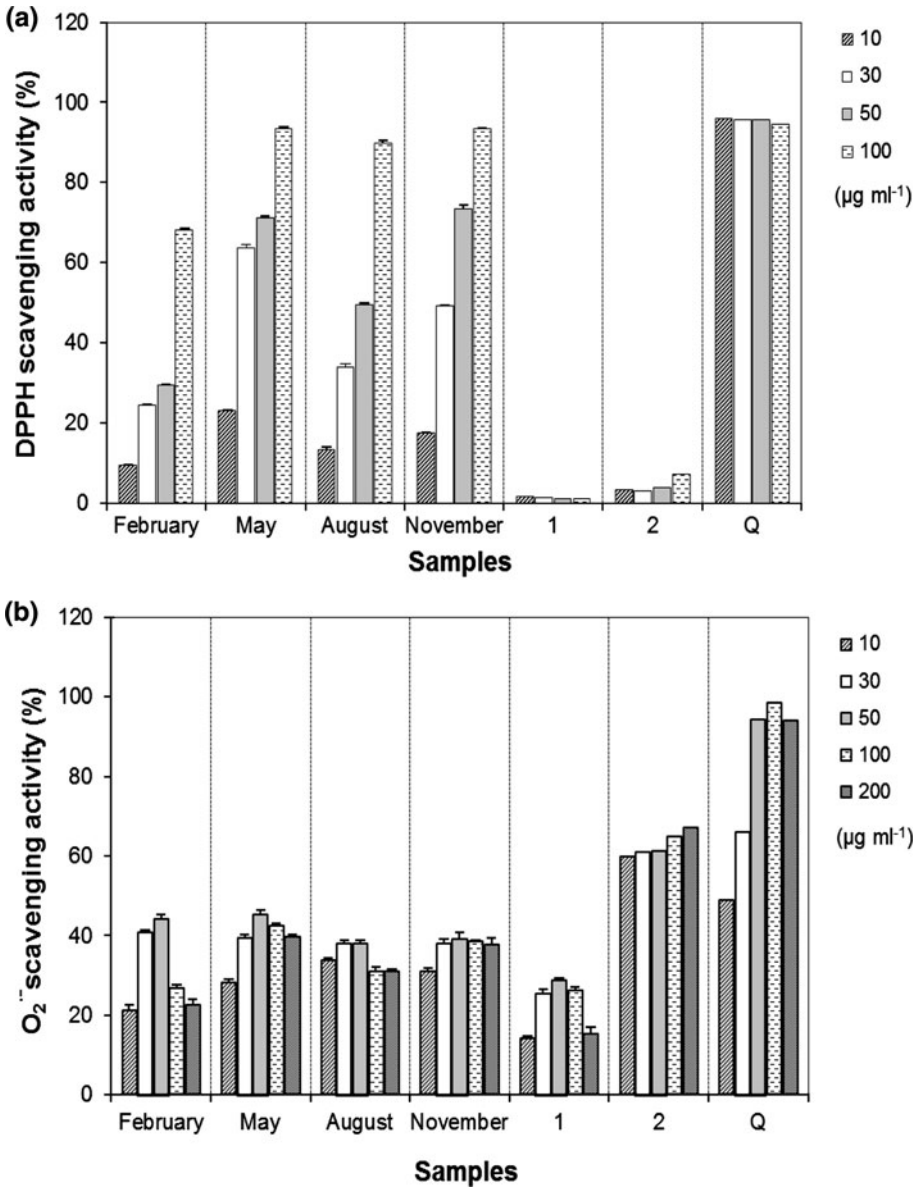

Fig. 2 DPPH radical (**a**) and superoxide radical (**b**) scavenging activities of licorice extract (LE) from *G. glabra* harvested at different harvest times, liquiritin (*1*) and glycyrrhizin (*2*). Quercetin (*Q*) was used as a reference compound. Data are the means of three replicates with standard deviations shown by *vertical bars*

times was not directly related to fluctuations in the global and UV radiation recorded.

Polyphenolic content

Polyphenols occur in all plant foods and contribute to the beneficial health effects of vegetables and fruit, due at least in part to their antioxidant properties (Parr and Bolwell 2000). The contents of TP, TF, and TT in LE significantly varied within different harvest times with the highest values in November, August and May, respectively (Table 1).

The variation pattern in the TP content of LE was in agreement with an earlier study with *Solanum lycopersicum* fruits (Toor et al. 2006). Our results could not confirm that phenolic compounds increase with increasing light intensity (Slimestad and Verheul 2009), because variations in the total content of polyphenols in LE across harvest times did not match up with the fluctuations in the global and UV radiation recorded. The lower contents of TP, TF and TT in the samples from February could be due to decreased active biosynthesis during cooler weather, as was previously remarked (Yao et al. 2005). It was further observed that the TP variation pattern was the reverse of that exhibited by liquiritin, but similar to that of glabridin (4) and glabrene (5). The variation pattern in the TF and TT contents of LE were consistent with earlier investigations on some leafy vegetables (Hertog et al. 1992) and leaves of *Betula pubescent* (Salminen et al. 2001), respectively. The content of TF (flavones and flavonols) at different harvest

Table 3 Concentration (μg ml^{-1}) response of antioxidant activity for licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times, by the β-carotene-linoleate bleaching method (at 180 min)

Parameters of antioxidant activity	Samples	Concentration (μg ml^{-1})		
		200	50	10
A_A	February	82.87 ± 2.63	40.99 ± 4.19	7.31 ± 0.47
	May	92.23 ± 0.34[ab]	78.72 ± 1.77[a]	20.63 ± 1.76[a]
	August	91.43 ± 0.30[ac]	78.05 ± 3.78[a]	17.75 ± 0.77[a]
	November	94.52 ± 1.20[bc]	88.80 ± 2.17[b]	31.23 ± 3.72
	Q	99.46 ± 0.39	93.60 ± 0.71[b]	75.92 ± 0.70
R_{OR}	February	0.18 ± 0.03	0.57 ± 0.06	0.93 ± 0.01
	May	0.08 ± 0.01[ab]	0.22 ± 0.02[a]	0.80 ± 0.02[a]
	August	0.09 ± 0.01[ac]	0.22 ± 0.04[a]	0.82 ± 0.01[a]
	November	0.06 ± 0.02[bc]	0.11 ± 0.02[b]	0.69 ± 0.03
	Q	0.01 ± 0.00	0.06 ± 0.01[b]	0.22 ± 0.01
C_{AA}	February	695.53 ± 38.53	229.37 ± 43.36	27.90 ± 0.60
	May	901.32 ± 23.43[a]	628.63 ± 33.58[a]	84.00 ± 12.19[a]
	August	865.56 ± 9.04[a]	625.81 ± 59.14[a]	75.54 ± 7.54[a]
	November	963.19 ± 16.81[b]	814.58 ± 10.06[b]	156.28 ± 6.04
	Q	1,005.62 ± 10.61[b]	876.72 ± 11.72[b]	571.55 ± 7.55

Values followed by the same letter in the same column for the same antioxidant activity parameter are not significantly different (Tukey's test, $P < 0.05$, $n = 3 \pm$ SD)

A_A, Antioxidant activity index; R_{OR}, oxidation rate ratio; C_{AA}, antioxidant activity coefficient; Q, quercetin

times varied inversely with respect to that of the flavanone liquiritin, showing that environmental factors associated with harvest times of licorice seem to have favored the biosynthesis of a particular class of flavonoids at the expense of another.

Free radical scavenging activity

DPPH free radical scavenging assay

The most favorable DPPH scavenging activity of LE was in May and November (Fig. 2a). This effect was in a concentration-dependent manner, which is in accordance with a prior study (Di Mambro and Fonseca 2005). Compounds 1 and 2 showed a negligible effect, which is consistent with an earlier study (Kato et al. 2008). The variation pattern in the DPPH effect correlated significantly with changes in the contents of TP, TT, 4 and 5 (Fig. 4a, c, e, f). In a previous study, compounds 4 (glabridin) and 5 (glabrene) scavenged 31 and 86 % of the DPPH radical within 30 min of incubation, respectively (Belinky et al. 1998a).

Superoxide radical scavenging assay

The superoxide anion radical ($O_2^{\cdot -}$) may contribute to aging and age-related diseases (Ishii and Ishii 2006). The effect of LE toward $O_2^{\cdot -}$ varied significantly across harvest times at a concentration range of 10–200 μg ml^{-1} (Fig. 2b). However, no effect dependent on concentration was observed. As the LE at 50 μg ml^{-1} showed the highest effect across harvest times, this concentration was used for the correlation analysis. LE at 50 μg ml^{-1} partially correlated with changes in the content of 1 and 2 across harvest times (Fig. 4d). An appreciable antiradical effect (about 63 %) of glycyrrhizin (2) followed by a weak effect (14–26 %) of liquiritin in the range of 10–100 μg ml^{-1} were also observed.

Antioxidant activity

Inhibition of β-carotene-linoleate bleaching

The antioxidant effects of LE on the β-carotene consumption varied across harvest times. The concentration–effect relationship for LE in the range 10–200 μg ml^{-1} at different harvest times is shown in Table 3. Kinetic of reaction is shown in Online Resource 3. In general, the highest antioxidant effect (A_A) of LE in the range of 10–200 μg ml^{-1} was in November (31–95 %) and the lowest was in February (7–83 %). Compounds 1 and 2 showed no antioxidant effect in this system (Figure not shown). Both the data for oxidation rate ratio (R_{OR}) and activity coefficient (C_{AA}) support the antioxidant activity index (A_A) of LE. The oxidation rate ratio bears an inverse

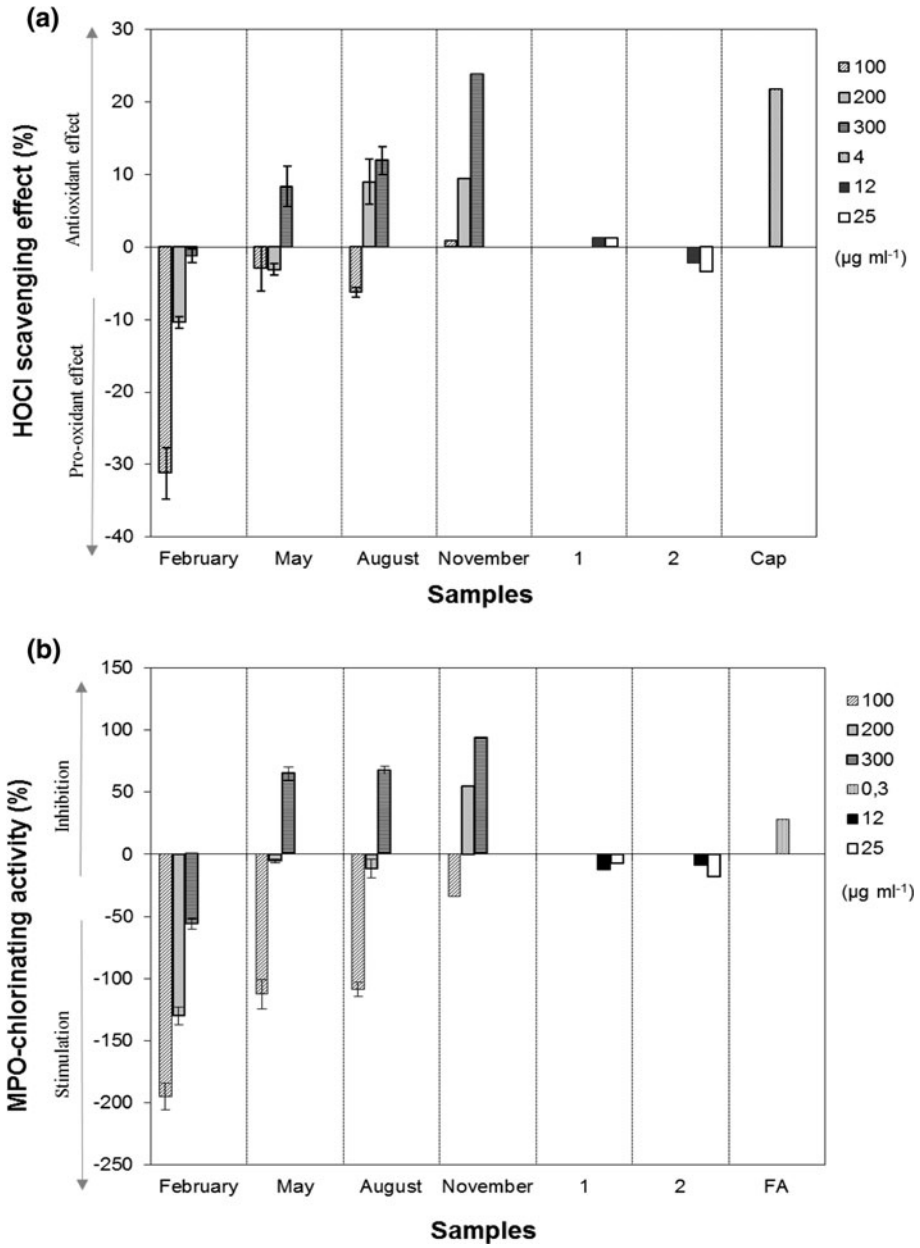

Fig. 3 Antioxidant activities of the licorice extract (LE) from *G. glabra* at different harvest times, liquiritin (*1*), and glycyrrhizin (*2*). Hypochlorous acid (HOCl) scavenging activity (**a**). Effect on the myeloperoxidase (MPO)-chlorinating system (**b**). Captopril (*Cap*) and flufenamic acid (*FA*) were used as reference compounds. Data are the means of three replicates with standard deviations shown by *vertical bars*

relationship with the antioxidant activity index. The activity coefficient increases directly with the increase in value of the antioxidant activity index. Variations in the β-carotene bleaching inhibition of LE across harvest times significantly correlated with changes in the contents of TP, TF, TT, 4 and 5 (Fig. 4a–c, e, f).

Recently, a licorice infusion at 200 µg ml^{-1} was shown to inhibit the β-carotene consumption by about 83 % (Cheel et al. 2010). In another investigation, a licorice ethanolic extract and glabridin, its major constituent, inhibited β-carotene consumption by 87 and 93 %, respectively (Vaya et al. 1997). Glabrene was reported to be more potent than glabridin as an inhibitor of LDL oxidation (Belinky et al. 1998b). It has been reported that the oxidation of LDL, which is thought to contribute to atherogenesis (Witztum and Steinberg 1991), starts after the depletion of its endogenous lipophilic antioxidants, such as β-carotene and vitamin E (Belinky et al. 1998a). From the present study, the November LE may prove to be of potential health benefit in protecting LDL-associated carotenoids.

Hypochlorous acid scavenging assay

The myeloperoxidase (MPO)-derived oxidant HOCl plays a role in tissue injury under inflammatory conditions

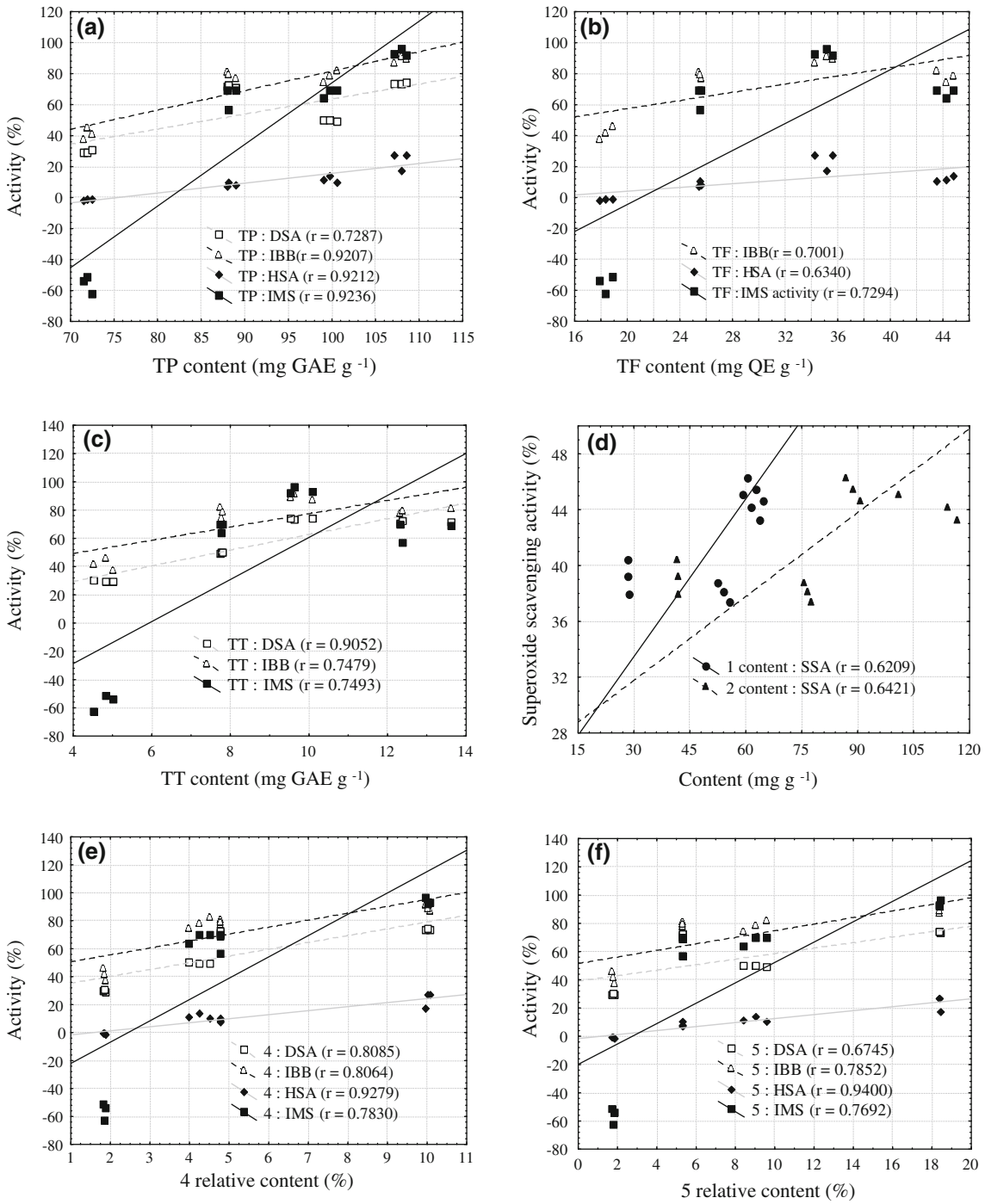

Fig. 4 Correlation analysis between the variations in the chemical content and the biological activities of LE from *G. glabra* at different harvest times. *TP* Total phenolics, *TF* total flavonoids, *TT* total tannins, *DSA* DPPH free radical scavenging activity, *SSA* superoxide anion radical scavenging activity, *IBB* inhibition of β-carotene-linoleate bleaching, *HAS* hypochlorous acid scavenging activity, *IMS* inhibition of myeloperoxidase-chlorinating system, (*1*) liquiritin, (*2*) glycyrrhizin, (*4*) glabridin and (*5*) glabrene. Pearson's correlation coefficient (*r*), $P < 0.05$

(Zhang et al. 2002). In the present investigation, the HOCl scavenging effect of LE in the range of 100–300 μg ml^{-1} significantly varied across harvest times with the highest effect being in November and the lowest in February (Fig. 3a). The May and August samples were antioxidant at higher concentrations, whereas they were oxidant at lower concentrations. Compounds 1 and 2 exhibited no meaningful activity against HOCl. Variations in the HOCl scavenging effects of LE across harvest times correlated significantly with changes in the contents of TP, TF, 4 and

Table 4 Effect of licorice extract (LE) from *Glycyrrhiza glabra* at different harvest times, liquiritin (1) and glycyrrhizin (2) on ethanol-induced gastric lesions in mice

Samples	Dose (mg kg^{-1})	Gastric lesions	
		Lesion index (mm)	Inhibition (%)
February	5	26.4 ± 1.4*	49.0
	25	10.3 ± 0.9*	80.1
	50	5.7 ± 1.1*	89.4
May	5	20.1 ± 1.2*	61.4
	25	9.1 ± 0.8*	82.0
	50	4.0 ± 0.6*	92.2
August	5	30.4 ± 1.3*	42.4
	25	16.0 ± 1.5*	69.3
	50	3.1 ± 0.4*	93.1
November	5	32.1 ± 1.6*	38.5
	25	12.7 ± 2.2*	71.2
	50	10.4 ± 1.7*	79.8
Lansoprazole	20	19.9 ± 1.1*	61.7
Control	–	52.0 ± 1.5	–
1	5	20.4 ± 1.6*	45.9
	10	14.7 ± 1.2*	61.0
	25	9.6 ± 1.0*	74.5
2	5	14.3 ± 1.4*	62.2
	10	6.7 ± 1.0*	82.5
	25	4.4 ± 0.8*	89.4
Lanzoprazole	20	14.3 ± 1.1*	62.1
Control	–	37.7 ± 2.1	–

Values are expressed as mean ± SEM. Analysis of variance followed by Dunnett's test

* $P < 0.01$ compared with control group

5 (Fig. 4a, b, e, f). Our results suggest that the traditional use of licorice against inflammatory processes could be favored when harvesting is in November.

Inhibition of myeloperoxidase-chlorinating system

Myeloperoxidase (MPO) is an enzyme (EC 1.11.1.7) linked to both inflammation and oxidative stress by its location in leukocytes and its role in catalyzing the formation of the highly chlorinating and oxidizing agent HOCl (Schindhelm et al. 2009). The effect of LE on the MPO-chlorinating activity varied significantly at different harvest times (Fig. 3b). In general, the samples from May, August and November displayed an inhibiting effect on MPO at higher concentrations and a stimulating effect (pro-oxidant effect) at lower concentrations. Compounds 1 and 2 also exhibited a pro-oxidant effect at 12 and 25 μg ml^{-1}. Variations in the effect of LE on the MPO system across harvest times significantly correlated with changes in the contents of TP, TF and TT, 4 and 5 (Fig. 4a–c, e, f). In a prior investigation, three herbal constituents of STW5 (Iberogast®) showed a pro-oxidant effect in the MPO-catalyzed chlorination system using the ACC–ethene model. Conversely, the ethanolic extract of *G. glabra*, another STW5 constituent, showed an antioxidant effect (Schempp et al. 2006). The present investigation suggests that the traditional use of licorice against inflammatory processes related with MPO could be favored when harvesting is in November.

Gastroprotective activity

The antiulcerogenic effect of licorice has been described by traditional and modern sources (Fiore et al. 2005). As can be seen in Table 4, the gastroprotective activity of LE at doses of 5, 25 and 50 mg kg^{-1} varied over harvest times in range of 38–61, 71–82 and 80–93 %, respectively. The May sample at the dose of 5 mg kg^{-1} displayed the most remarkable effect, while the lowest effects were in August and November. The February and May samples at a dose of 25 mg kg^{-1} exerted the most favorable effects. All samples at a dose of 50 mg kg^{-1} almost completely inhibited ethanol-induced gastric mucosal lesions. The compounds 1 and 2 (5–25 mg kg^{-1}) reduced the formation of gastric lesions by 46–89 %. Variation patterns in the gastroprotective activity of LE during harvest times could not be associated with changes in its chemical contents. The biological effects observed may be the result of complex interactions of synergism, additivity or even antagonism among LE constituents. In a prior study, a significant antigastric ulcer effect of water extract of licorice in rats was attributed to liquiritin apioside (Nakamura et al. 2003).

Conclusions

The content of individual components and grouped polyphenols of LE varied at different harvest times, and these changes were partially correlated with fluctuations in the antioxidant and antiradical activity. In general, the LE from May and November showed the most favorable antiradical and antioxidant effects, while the best gastroprotective effect was achieved in May. Liquiritin and glycyrrhizin appeared to contribute to the superoxide radical scavenging and gastroprotective effects, whereas glabridin and glabrene mainly accounted for the antioxidant and DPPH scavenging activity of LE. It is noteworthy that the total content of polyphenols could only represent a general pattern in LE and do not precisely reveal the true patterns of individual phenols over harvest times. Therefore, the variations of the content of individual compounds could be a better predictor of the changes in the bioactivities of

licorice. The data emerging from the present study represent an approach to determine the best time for harvesting licorice with optimal chemical and pharmacological properties. Further studies are recommended to perform greenhouse experiments at controlled growing conditions in order to compare the influence of light, temperature, precipitation (rainfall) and biotic agents.

Author contribution José Cheel and Lenka Tůmová (extract preparation, antioxidant and free radical scavenging assays, HPLC–DAD analysis, polyphenols determinations, HPLC quantification); Pierre Van Antwerpen and Jean Nève (inhibition of myeloperoxidase, HOCl scavenging assay and LC–TOF–MS analysis); Karim Zouaoui-Boudjeltia (obtention of recombinant myeloperoxidase); Carlos Areche and Aurelio San Martin (gastroprotective activity); Ivan Vokřál and Vladimír Wsól (HPLC–MS analysis); Jarmila Neugebauerová (cultivation and harvesting of licorice).

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Conflict of interest The authors declare that they have no conflict of interest.

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